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Pharmacology of (2*S*,4*Z*)-*N*-[(2*S*)-2-Hydroxy-2-phenylethyl]-4-(methoxyimino)-1-[(2′-methyl[1,1′-biphenyl]-4-yl)carbonyl]-2-pyrrolidinecarboxamide, a New Potent and Selective Nonpeptide Antagonist of the Oxytocin Receptor

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ABSTRACT

We have discovered a new, potent, selective, and orally active oxytocin receptor antagonist, (2*S*,4*Z*)-*N*-[(2*S*)-2-hydroxy-2-phenylethyl]-4-(methoxyimino)-1-[(2′-methyl[1,1′-biphenyl]-4-yl)carbonyl]-2-pyrrolidinecarboxamide (compound **1**). We report the biochemical, pharmacological, and pharmacokinetic characterization in vitro and in vivo of this compound. Compound **1** competitively inhibits binding of [^{3}H]oxytocin and the peptide antagonist ^{125}I-ornithine vasotocin analog to human and rat oxytocin receptor expressed in human embryonic kidney 293-EBNA or Chinese hamster ovary cells with nanomolar potency. Selectivity against vasopressin receptor subtypes is >6-fold for V1a and >350-fold for V2 and V1b. Compound **1** inhibits oxytocin-evoked intracellular Ca^{2+} mobilization (IC_{50} = 8 nM). Compound **1** has no intrinsic agonist activity at the oxytocin receptor. Oxytocin-induced contraction of isolated rat uterine strips is blocked by compound **1** (pA_2 = 7.82). In anesthetized nonpregnant rats, single administration of compound **1** by i.v. or oral routes causes dose-dependent inhibition of contractions elicited by repeated injections of oxytocin with ED_{50} = 3.5 mg/kg i.v. and 89 mg/kg p.o., respectively. Compound **1** significantly inhibits spontaneous uterine contractions in pregnant rats near term when administered intravenously or orally. We conclude that compound **1** is a potent, selective, and orally active nonpeptide oxytocin receptor antagonist, which is a suitable candidate for evaluation as a potential tocolytic agent for the management of preterm labor.

Premature birth is a major problem in obstetrics affecting about 10% of all births and being the largest cause of perinatal morbidity and mortality. The impact on society is significant in terms of costs of neonatal intensive care and for the emotional and social stress to the family. The physiopathology of human preterm labor is complex and multifactorial. Preterm increase of uterine activity is a common complication of pregnancy and accounts for many cases of preterm labor. Pharmacological interventions aimed at maintaining uterine quiescence (tocolysis) have been, and are likely to remain, the cornerstone of pharmaceutical management of preterm labor. However, current tocolytic agents (β-mimetics, magnesium sulfate, calcium channel blockers, or prostaglandin synthesis inhibitors) suffer from a minimal effectiveness and show important fetal and maternal side effects. Therefore, it is obvious that a safe and effective oral treatment delaying spontaneous preterm birth would have tremendous clinical benefits.

The peptide hormone oxytocin (OT) is a potent contractor of the human uterus. OT mediates its effect through activation of the G protein-coupled oxytocin receptor (OT-R) that is expressed in myometrial cells. OT-R is coupled to phospholipase C activation, leading to intracellular synthesis of inositol phosphates and mobilization of calcium. In turn, the rise in intracellular calcium concentration promotes a cascade of events, including phosphorylation of myosin, that then acts on actin and induces uterine muscle cell contraction. Before onset of labor and in the term myometrium, the OT-R density

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ABBREVIATIONS: OT, oxytocin; OT-R, oxytocin receptor; OVTA, ornithine vasotocin analog; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; CHO, Chinese hamster ovary; HEK, human embryonic kidney; PBS, phosphate-buffered saline; AVP, arginine vasopressin; AUC, area under the curve; SD, Sprague-Dawley; FLIPR, fluorescence imaging plate reader; PE, polyethylene; ANOVA, analysis of variance.

increases dramatically (Fuchs et al., 1982; Maggi et al., 1990). The onset of labor is not associated with an increase in levels of circulating OT and, therefore, the exact role of OT remains controversial. However, there is some indication that OT is produced locally in the uterus (Chibbar et al., 1993). So, it seems clear that during parturition (term and preterm) OT is a vital mediator of uterine contractile activity. OT and OT-R synthesis is highly regulated and probably involves the interplay of many factors, including estrogens, progesterone, prostaglandins, bacterial endotoxin, and cytokines (Novy and Liggins, 1980; Fuchs and Fuchs, 1984). Therefore, the most direct way to determine the relative importance of oxytocin in parturition is to block its effects pharmacologically (Akerlund et al., 1987). Accordingly, OT-R antagonists would have a therapeutic value in the management of many cases of preterm labor for their suppressive effects on enhanced uterine contractility. Recently, the injectable peptide OT-R antagonist atosiban has shown efficacy for imminent preterm birth. However, it is inconvenient to use, because it requires constant infusion and is limited to the short-term treatment (1–2 days) of the acute phase of preterm labor (Moutquin et al., 2000; Romero et al., 2002). This drug also has activity against the V1a receptor. It is suggested that more specific inhibitors may be more efficacious (Wilson et al., 2001).

In this article, we report on the discovery of (2*S*,4*Z*)-*N*-[(2*S*)-2-hydroxy-2-phenylethyl]-4-(methoxyimino)-1-[(2′-methyl[1,1′-biphenyl]-4-yl)carbonyl]-2-pyrrolidinecarboxamide, compound **1**, a new potent and selective nonpeptide low molecular weight OT-R antagonist. Here, we describe the biochemical and pharmacological characterization of compound **1**. The in vitro properties of compound **1** were investigated using cellular preparations expressing the rat or human OT-R. The tocolytic effect of compound **1** was studied using rat models of uterine contractions. Part of the present data was presented at the 49th Annual Meeting of Society for Gynecologic Investigation (Chollet et al., 2002) and at the 17th European Congress of Obstetrics and Gynaecology (Gillio Tos et al., 2002).

Materials and Methods

Materials

Compound **1** (Fig. 1) was chemically synthesized at the Serono Pharmaceutical Research Institute (Geneva, Switzerland). The chemical structure of compound **1** was determined by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, and infrared spectrometry. The purity assessed by high-pressure liquid chromatography and C, H, N elemental analysis was >98%. Atosiban [1-(3-mercaptopropanoic acid)-2-(*O*-ethyl-D-tyrosine)-4-L-threonine-8-L-ornithine-oxytocin] was from Neosystem (Strasbourg, France). [^{3}H]Oxytocin radioligand (44 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA). The radioligand ^{125}I-ornithine vasotocin analog (^{125}I-OVTA; 2,200 Ci/mmol) was from PerkinElmer Life Sciences. In all the in vivo experiments, compound **1** was vehicled in 5% *N*-methylpyrrolidone, 25% polyethyleneglycol 200, 30% polyethylene glycol 400, 20% propylene glycol, and 20% saline), whereas ritodrine was solubilized in saline.

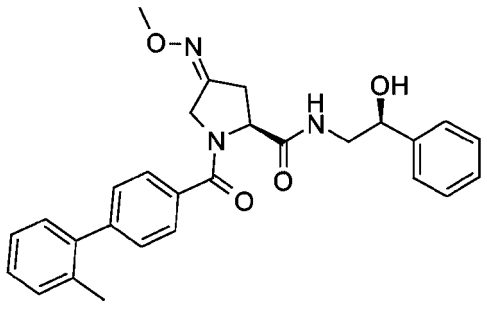

Fig. 1. Chemical structure of compound **1**.

Oxytocin, ritodrine, urethane, diethylstilbestrol, and (5*Z*,9α,11α,13*E*,15*S*)-9,11,15-trihydroxyprosta-5,13-dienoic acid (prostaglandin $F_{2\alpha}$; $PGF_{2\alpha}$) were supplied by Sigma-Aldrich (St. Louis, MO). Fluo-4 dye was purchased from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium-F-12 cell culture medium was supplied by Invitrogen (Paisley, UK). Acebutol was supplied by Sigma-Aldrich (Milan, Italy). Acetonitrile, formic acid, *tert*-metylbutylether, ammonium formate, and all analytical or LiChrosolv grade reagents were supplied by Fluka (Buchs, Switzerland).

In Vitro Experiments

Cell Culture. CHO cell lines expressing the vasopressin receptor subtypes, the human or the rat oxytocin receptor were maintained in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented fetal calf serum, 4 mM glutamine, and 500 units/ml penicillin and streptomycin, in an atmosphere of 95% air and 5% CO_2 at 37°C (Terrillon et al., 2002).

HEK293-EBNA cell line expressing the human oxytocin receptor was maintained in Dulbecco's modified Eagle's medium-F-12 (1:1) supplemented with 10% fetal calf serum and 300 μg/ml hygromycin B, in an atmosphere of 95% air and 5% CO_2 at 37°C. In HEK293-EBNA cells, B_{max} was 1900 ± 700 fmol/mg of protein (n = 2) and K_D for [^{3}H]OT was 1.1 ± 0.1 nM (n = 2). These values were comparable to previous reports (Jasper et al., 1995).

Membrane Preparation. Culture dishes of CHO cells expressing one receptor subtype were washed twice with PBS without Ca^{2+} and Mg^{2+}. Lysis buffer (15 mM Tris-HCl, 2 mM $MgCl_2$, and 0.3 mM EDTA, pH 7.4) was added and cells were scraped, polytron-homogenized, and centrifuged at 100*g* for 5 min. The supernatant was then centrifuged at 44,000*g* for 20 min at 4°C. Pellets were resuspended in buffer A (50 mM Tris-HCl and 5 mM $MgCl_2$, pH 7.4) and centrifuged a second time at 44,000*g* for 20 min at 4°C. Pellets were then resuspended in the appropriate volume of buffer A. Protein concentration was estimated using the DC protein assay method (Bio-Rad, Hercules, CA). Membranes were immediately used or aliquoted and stored in liquid nitrogen.

HEK293-EBNA cells expressing the human OT-R were detached from the culture dish with PBS, EDTA 1 mM and then washed twice with PBS without Ca^{2+} and Mg^{2+}. The pellet was resuspended in lysis buffer (15 mM Tris-HCl, 2 mM $MgCl_2$, and 5 mM EDTA, pH 7.4), homogenized (Dounce homogenizer), and centrifuged at 250*g*, 10°C for 10 min. The pellet was discarded. The supernatant was then centrifuged at 40,000*g* for 60 min at 4°C. Pellets were resuspended in buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, and 0.1% bovine serum albumin, pH 7.4), broken down again with the Dounce homogenizer, and centrifuged at 250*g* for 5 min at 4°C. The pellet was discarded. Protein concentration was estimated using the DC protein assay method (Bio-Rad). Membranes were immediately used or aliquoted and stored at −80°C.

Binding Experiments. Binding experiments on CHO cell membranes were performed as described previously (Terrillon et al., 2002). Briefly, affinities of the various ligands for the vasopressin and OT-R subtypes were determined by competition experiments using 200 to 250 pM ^{125}I-OTA antagonist (^{125}I-d(CH_2)$_5$ [Tyr(Me)^2,Thr^4,Orn^8,Tyr (NH_2)^9]VT; Elands et al., 1988) for OT receptor, 40 to 60 pM [^{125}I]HO-LVA antagonist (^{125}I-HO-Phaa,D-Tyr(Me),Phe,Gln,Asn,Arg,Pro,Arg (NH_2); Barberis et al., 1995) for the V_{1a} vasopressin receptor, and 1 to 3 nM [^{3}H]AVP (for V_{1b} and V_2 vasopressin receptors) as radioligands. Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. Concentrations of OT-R antagonists varied from 100 pM to 10 μM. Membranes were

used at 0.5 to 1.5 μg of protein/assay for competition with iodinated ligands and 5 to 10 μg of protein/assay with [^{3}H]AVP. Binding assays were performed at 30°C for 1 h. Ligand binding data were analyzed with the computer program Ligand, and the inhibition constants, K_i, were determined with a nonlinear least-squares regression from at least three independent competition experiments, each performed in triplicate.

Competition binding experiments on HEK293-EBNA membranes expressing human OT-R and using [^{3}H]OT at 1 nM were performed by incubating varying concentrations of oxytocin antagonists from 100 pM to 10 μM in a volume of 200 μl of buffer A containing 1% dimethyl sulfoxide for 1 h at 20°C in Corning NBS 96-well plates. Nonspecific binding was determined in the presence of 1 μM OT. The reaction was stopped by filtration through GF/C glass fiber unifilter plates (catalog no 7700-4301; Whatman, Maidstone, UK) that were presoaked in 0.5% polyethylenimine (catalog no. P-3143; Sigma-Aldrich). Unifiltered plates were then washed four times with 200 μl of buffer A without bovine serum albumin and dried for 30 min at 50°C. The bottom of the plates was sealed with tape (1450-461; PerkinElmer Life Sciences), 100 μl of scintillation fluid (Ultima Gold XR; Packard 6013119; PerkinElmer Life Sciences) was added, the plates were sealed on top, incubated for 1 h at 20°C, and then counted with a Microbeta 1450 plate counter (PerkinElmer Wallac, Gaithersburg, MD).

Competition binding experiments on HEK293-EBNA membranes expressing human OT-R and using ^{125}I-OVTA antagonist at 0.1 nM were performed by incubating for 30 min at 20°C in Corning NBS 96-well plates containing various concentrations of OT-R antagonists (10 pM–10 μM), 10 to 20 μg/ml cell membranes, and 1 mg/ml PVT-PEI wheat germ agglutinin-coupled type A SPA beads (RPNQ0003; Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK) in a final volume of 100 μl of buffer A containing 1% dimethyl sulfoxide. Nonspecific binding was determined in the presence of 1 μM oxytocin. The plates were then counted with a Microbeta 1450 plate counter (PerkinElmer Wallac). The data were analyzed by the software Prism 3.0 (GraphPad Software Inc., San Diego, CA).

Intracellular Ca^{2+} Measurements. HEK293-EBNA cells stably transfected with the human OT-R were cultured to subconfluence, collected, and seeded at 10^5 cells/well in a 96-well plate precoated with poly-L-lysine. The plate was cultured for 24 h and the cells were loaded with 4.5 μM Fluo-4 dye in Dulbecco's modified Eagle's medium-F-12 medium without fetal calf serum for 1 h at 37°C. Increasing concentrations of oxytocin antagonist compounds (compound **1** or atosiban) were added to cells and incubated for 30 min. Cells were washed in fluorescence imaging plate reader (FLIPR) buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM glucose, pH 7.4), and increasing concentrations of antagonist compounds were added again to the cells. Intracellular calcium concentration was measured for 1 min (excitation at 488 nm, emission at 510–570 nm) using a FLIPR instrument (Molecular Probes). Cells were then stimulated with 10 nM OT, and transient intracellular calcium mobilization was recorded for 4 min. The OT EC_{50} value was 5 nM for these cells. Ca^{2+} concentration was expressed in percentage of maximal response elicited by OT. IC_{50} measurements were calculated by analysis of the data using Excelfit software (ID Business Solution Ltd., Guilford, Surrey, UK).

Ex Vivo Contraction Experiments. Uteri were removed from pregnant Wistar rats (Iffa Credo, L'Arbresele, France) at 21 days postconception and longitudinal myometrial segments were prepared. Each muscle strip (2 × 10 mm) was suspended vertically in an organ bath (5 ml, 32°C) containing Krebs' solution (118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11 mM glucose) gassed with an atmosphere composed of 95% O_2 and 5% CO_2. The muscle strips were loaded at 1 g as an initial tension and allowed to equilibrate for 90 min and to establish a steady spontaneous contractile activity. They were then stimulated with 80 mM high-K^+ solution for 3 min, and allowed to equilibrate again for 45 min, before the OT-R antagonist compound **1** or atosiban was added at a concentration of 10^{-8} to 10^{-6} M. After 30 min, each muscle strip was subjected to cumulative additions of OT (10^{-11}–10^{-6} M) at 5-min intervals. OT-induced contractions were analyzed as the area between the contraction curve and baseline (area under the curve, AUC) during a 5-min period after application of OT. The change in contractile activity was expressed as percentage of the AUC of 80 mM high-K^+-induced contraction. The EC_{50} values and maximal contractions were estimated from the concentration-response relationships. Antagonist potencies (pA_2) were determined according to the procedure of Arunlakshana and Schild (1959).

In Vivo Experiments

Oxytocin- or $PGF_{2\alpha}$-Induced Uterine Contractions in Anesthetized Nonpregnant Rats. Oxytocin-induced uterine contractions were produced according to Clineschmidt et al. (1991). Briefly, nonpregnant Sprague-Dawley CD (SD) BR female rats (Charles River Italica, Calco, Italy), weighing 200 to 300 g, were used. They received an i.p. injection of 250 μg/kg diethylstilbestrol 24 and 18 h before the experiment. On the day of the experiment, they were anesthetized with urethane (1.05 g/kg i.p.) and placed on a homeothermic operating table. A midline incision at the hypogastrium level was made, one uterine horn exposed, and its tubal-end closed (near the ovary) by a ligature with surgical silk. About 3 cm posterior to the first tie, the uterine horn wall was incised (close to the uterus body) and PE240 tubing was inserted into the lumen and secured with surgical silk. After filling the internal cavity with 0.2 ml of sterile physiological saline solution, the catheter was connected to an amplifying/recording system (MacLab; ADInstruments Pty Ltd., Castle Hill, Australia) via a P23ID Gould Statham pressure transducer. One jugular vein was then isolated and cannulated with a PE60 catheter connected to a butterfly needle for the i.v. administration of oxytocin, compound **1**, or ritodrine. To allow oral administrations, the esophagus was cannulated with a PE90 cannula. A carotid artery was also cannulated with a PE50 cannula to collect blood samples for successive analytical determinations of plasma levels of compound **1**.

After a suitable stabilization period, repeated bolus i.v. administrations of OT at 0.5 μg/kg were performed every 35 min for a total of nine times. Five minutes before the fourth injection, compound **1** (during a 5-min infusion i.v. or p.o.) or ritodrine (bolus i.v. or p.o.) was administered.

Blood samples from compound **1**-treated rats were collected at 0.033, 0.5, 1.5, and 3.5 h after 0.3, 1, 3, 10, and 30 mg/kg (i.v. route) and at 0.5, 1, 2, and 3.5 h after 3, 10, 30, and 60 mg/kg (oral route). Plasma samples were then obtained using routine laboratory procedures and stored at −70°C.

In separate experiments, uterine contractions were induced by prostaglandin F_{2a} administrations at 100 μg/kg (during a 10-min i.v. infusion). After three basal injections and 5 min before the fourth injection of $PGF_{2\alpha}$, increasing doses of compound **1** or ritodrine 10 mg/kg were administered i.v. The overall experimental design was the same used for the contractions induced by oxytocin.

The contractile responses to either OT or $PGF_{2\alpha}$ were quantified by measuring the AUC of the changes in intraluminal uterine pressure (by Chart version 4.04 for Windows software; ADInstruments Pty Ltd.) over the whole 35-min period, in the case of OT-induced contractions, and for the first 15 min postinjection period, in the case of $PGF_{2\alpha}$-induced contractions. Percentage of variations of AUCs determined after each oxytocin or $PGF_{2\alpha}$ administration were calculated in comparison with the AUC obtained with the third agonist injection (set as 100%). The effect of compound **1** or ritodrine was expressed at each time point as the percentage of inhibition of the above-mentioned variation values after the administration of each dose of test compound compared with that obtained at the corresponding time point in the group receiving the vehicle alone. From the inhibition values obtained for each dose group at the peak effect, a dose-response curve was plotted and, when possible, the relative ED_{50} value calculated (by S-Plus 2000 version 4.6 statistical soft-

ware; Mathsoft, Inc., Seattle, WA). Statistical differences between treatment groups at each time-point were determined by using one-way ANOVA followed by Tukey's test.

Spontaneous Uterine Contractions in Anesthetized Late-Term Pregnant Rats. Late-term pregnant (certified at days 19–21 of pregnancy) SD CD BR female rats (Charles River Italica), weighing 350 to 400 g, were anesthetized with urethane (1.05 g/kg i.p.) and placed on a homeothermic operating table. A midline incision at the hypogastrium level was made, one pregnant uterine horn exposed, and its tubal end (near the ovary) was closed by a ligature with surgical silk.

Corresponding to the fetus close to the ovary, the uterine-horn wall was incised, taking care not to injure the adjacent placenta, and PE240 tubing with a latex balloon (9-mm length when empty, capacity 0.1 ml; Radnoti, Monrovia, CA) on the top was inserted into the lumen and secured to the uterine wall with surgical silk. After filling the internal cavity of the latex balloon with 0.1 ml of sterile physiological saline solution, the catheter was connected to an amplifying/recording system (MacLab; ADInstruments Pty Ltd.) via a P23ID Gould-Statham pressure transducer. One jugular vein was isolated and cannulated with a PE60 polyethylene cannula for the i.v. administration. After the surgical preparation, a 30-min stabilization period was observed and then the effects of ritodrine or increasing doses of compound **1** (given as 10-min i.v. infusion, bolus i.v. or p.o.) were evaluated by measuring the resulting uterine contractions.

For the i.v. administration (infusion or bolus) the uterine contractile activity was quantified by calculating the AUC during the 10-min injection period. The percentage of variation of the AUC values relative to the spontaneous uterine response observed after each compound administration was calculated in comparison with the value recorded before the first dose administration (basal value). The effect of compound **1** or ritodrine was evaluated by comparing pre- and post-treatment luminal uterine pressure values. For the oral administration, the same computation procedure was applied at different time points after treatment.

Statistical differences between treatment groups at each time point were determined by using one-way ANOVA followed by Tukey's test.

Pharmacokinetics of Compound 1 in Female Rat. Female nonpregnant SD CD BR rat (Charles River Italica), weighing 200 to 300 g, were used. Compound **1** was administered in groups of three animals i.v. at a single dose 10 mg/kg or orally at a single dose of 50 mg/kg. Blood samples from compound **1**-treated rats were collected at 0.083, 0.5, 1.5, 4, 6, and 24 h for i.v. route and at 0.5, 1, 2, 4, 8, 24, 30, and 48 h after oral route. Plasma samples were then obtained using routine laboratory procedures and stored at −70°C before analytical determination (see protocol below). AUC values were calculated up to the last time point by the trapezoidal rule. The absolute oral bioavailability (F) was calculated by the ratio of AUC (oral) over AUC (i.v.) corrected for the dose. The half-life ($t_{1/2}$) after i.v. administration was measured graphically in the 1.5- through 24-h range.

Analytical Determinations of Plasma Levels of Compound 1. Compound **1** levels were monitored in pooled plasma samples (n = 6–7 for each dose) obtained from the oxytocin-induced uterine contractions in anesthetized nonpregnant rat experiment or in pooled plasma samples (n = 3) obtained from pharmacokinetic experiment in conscious nonpregnant rat. Analyses were carried out on 0.2 ml of plasma alkalinized with 30 μl of 30% aqueous ammonia solution and spiked with 20 μl of acebutol (10 μg/ml methanol). The extraction procedure was performed with 1.5 ml of *tert*-methylbutylether by whirl mixing for 20 s, twice. After centrifugation, the organic layer was transferred and taken to dryness in a rotary evaporator apparatus (SC210A SpeedVac Plus; Thermo Savant, Rome, Italy) and the residue solubilized in 30 μl of acetonitrile plus 170 μl of 0.1% aqueous formic acid. An API 365 (Applied Biosystems, Monza, Italy) mass spectrometer, equipped with a Turbo Ion Spray source set in positive ion monitoring mode and connected to a 1100 Series binary pump high-performance liquid chromatography (Agilent, Milan, Italy), was used. Separation of compound **1** and acebutol (internal standard) was achieved on a security guard C18 (4 × 2-mm i.d.) column connected to a Luna C_{18} (30 × 2-mm i.d., 5 μm) analytical column (both from Phenomenex, Bologna, Italy). Compounds were eluted by means of a binary gradient consisting of 0.1% aqueous formic acid solution (A) and acetonitrile (B). The gradient started with 85:15 (v/v) A/B delivering the solvents at a flow rate of 0.3 ml/min and reaching the 50:50 (v/v) ratio within 0.8 min and then 10:90 (v/v) ratio within 0.1 min, maintained for 0.25 min. Compounds were monitored by multiple reaction monitoring acquisition scan mode, according to the fragmentation pathway 472.2 amu (Q1) to 195.1 amu (Q3) for compound **1** and 337.1 amu to 116.2 for acebutol. Other main instrumental setting parameters were as follows: ion source voltage, 5500 V; source temperature, 350°C; orifice voltage, 36 V; ring voltage, 200 V; collision energy, 29 V; and cell electron multiplier, 2200 V. Injection of the samples (10 μl) was by means of an autosampler (HTS PALsystem; CTC Analytics, Zwingen, Switzerland). A linear response was obtained in the calibration range 1 to 1000 ng/ml (n = 11; correlation = 0.9876). Intraday assay accuracy was in the range 2.5 to 7.2% (as coefficient of variation) and precision in the range from −10.6 to 11.2%. The limit of quantitation was set at 1 ng/m1.

Results

Affinities of Compound 1 for Oxytocin and Vasopressin Receptors and Selectivity Profile. Compound **1**, atosiban, and the natural hormone oxytocin were assayed for binding affinity to human OT-R by competitive binding analysis with either the [^{3}H]OT agonist or the ^{125}I-OVTA antagonist using HEK293-EBNA cells (Fig. 2, A and B). Likewise, compound **1**, atosiban, and OT were assayed for displacement binding of radiolabeled ligands to human or rat OT-R and the three human vasopressin receptor subtypes V1a, V1b, and V2 in CHO cells. The results are summarized in Table 1. Compound **1** showed a high affinity for OT-R, comparable with the peptide antagonist atosiban, albeit lower than OT. Unlike atosiban, compound **1** was very selective against V1a (>6-fold), V1b (>350-fold), and V2 (>350-fold). Moreover, compound **1** exhibited a 5-fold better affinity for human OT-R than for rat OT-R. The high selectivity profile of compound **1** was further demonstrated in binding assays against a panel of 50 various receptors, enzymes, and ion channels. Tested at 1 μM, compound **1** did not interact with receptors of peptide ligands (angiotensin, bradykinin, cholecystokinin, endothelin, chemokine, luteinizing hormone releasing hormone, neurokinin, neuropeptide Y, and opiates), nonpeptide ligands (adenosine, adrenergic, dopamine, histamine, acetylcholine, serotonin, purine, steroid hormones, prostaglandin, and inositol phosphate), various enzymes (cyclooxygenases, phospholipases, adenylate cyclase, aldose reductase, elastase, acetylcholine esterase, human immunodeficiency virus protease, phosphodiesterases, and protein kinase C), or ion channels (calcium, sodium, potassium, *N*-methyl-D-aspartate kainate) (data not shown). Compound **1** inhibited phosphodiesterase IV with IC_{50} = 6.1 μM, a value about 300-fold higher than the affinity for OT-R. Thus, compound **1** showed a very clean selectivity profile with specific interaction with OT-R.

Antagonist Properties of Compound 1 in HEK293-EBNA Cells Expressing the Oxytocin Receptor. The functional characterization of compound **1** on human OT-R was performed in HEK293-EBNA cells. Compound **1** was able to dose dependently inhibit the mobilization of intracellular calcium elicited by activation of OT-R with OT (Fig. 3). In this assay, compound **1** (IC_{50} = 8 nM) was more potent

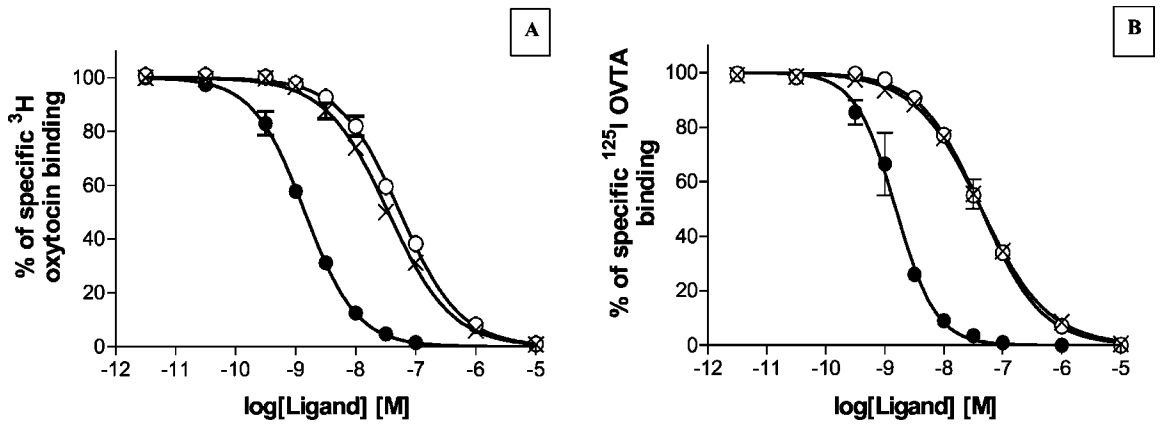

Fig. 2. Competitive displacement binding assay of the radiolabeled agonist [^{3}H]OT (A) or the antagonist ^{125}I-OVTA (B) in HEK293-EBNA cells transfected with the human OT-R (receptor density = 2.0 pmol/mg of membrane protein) by oxytocin (●), compound **1** (X), or atosiban (○). Each point represents the mean ± S.E. of three independent experiments, each performed in triplicate.

TABLE 1
Binding affinity of compound **1** and atosiban for oxytocin and vasopressin receptors

K_i	Compound **1**	Atosiban	Oxytocin
nM			
Human OT-R/CHO	28	81	0.5
Human OT-R/HEK293-EBNA	17	27	0.71
Human V1a/CHO	170	3.5	123
Human V1b/CHO	>10,000	657	1,782[a]
Human V2/CHO	>10,000	954	1,544
Rat OT-R/CHO	135	76	1.9

[a] Thibonnier et al., 1997.

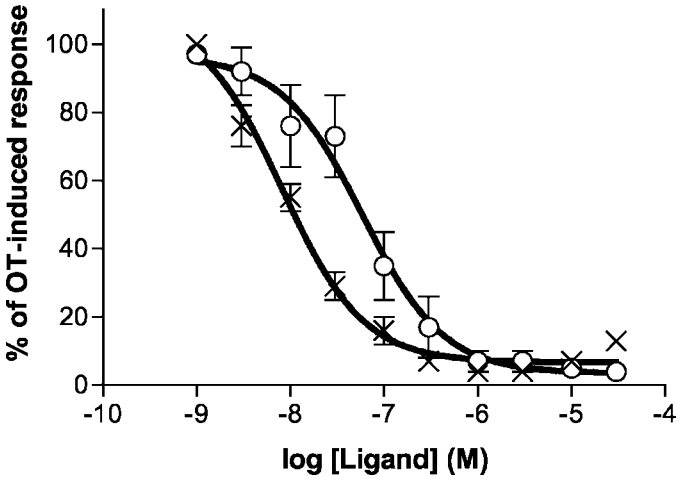

Fig. 3. Inhibitory effect of compound **1** (X) and atosiban (○) on the intracellular mobilization of Ca^{2+} stimulated by oxytocin in HEK293 cells transfected with the human OT-R. Calcium mobilization was measured by FLIPR using the fluorescent dye Fluo-4. Data are presented as the mean ± S.E. of four independent determinations.

than atosiban (IC_{50} = 59 nM). When added alone to OT-R/HEK293-EBNA cells, compound **1** tested up to 10 μM did not induce any release of calcium, indicating that the compound is devoid of any agonist activity.

Effect of Compound 1 on Oxytocin-Induced Contractions of Rat Uterine Tissue. Functional antagonism of compound **1** was further investigated using isolated rat uterine segments mounted on an organ bath. Compound **1** blocked the contraction of the uterine muscle strips explants from pregnant animals evoked by increasing concentrations of OT with pA_2 = 7.82 (Fig. 4).

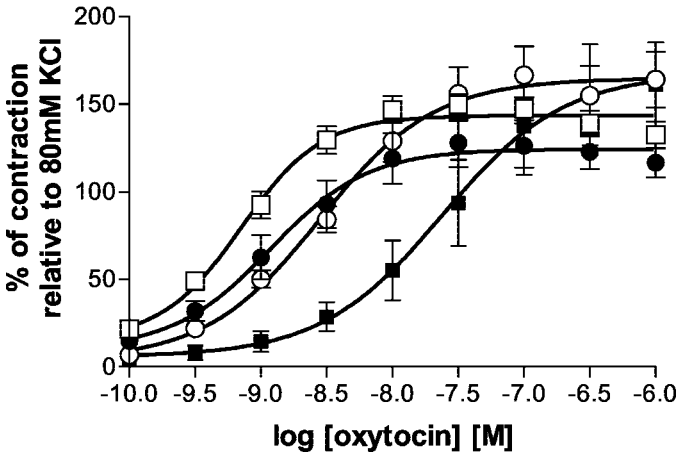

Fig. 4. Effect of compound **1** in uterine tissue. Uteri were removed from Wistar rats at day 21 postconception. The OT-induced contractions of longitudinal myometrial segments were examined in the absence (□) or presence of compound **1** at 10^{-8} M (●), 10^{-7} M (○), or 10^{-6} M (■). The amplitude of contraction was expressed as percentage of 80 mM high-K^+-evoked responses. The EC_{50} values and maximal contraction were estimated from the concentration-response curves as the mean of three independent experiments. In any experiment, each curve was established on three separate strips from three different animals so that each point is the mean of nine determinations.

Effect on Oxytocin-Induced Uterine Contractions in Anesthetized Nonpregnant Rats. Each administration of OT induced a train of uterine contractions, recorded as variations in the intraluminal pressure, of variable frequency and intensity that gradually declined in the 35-min postinjection period. Repeated injections of OT resulted in reproducible uterine contractile responses. No major changes over time were observed when administering the vehicle alone (data not shown). Figure 5 reports a representative tracing of the inhibitory effect obtained with compound **1** at 30 mg/kg i.v. The effect was very marked and persisted over time. Administration of increasing doses of compound **1** by i.v. (0.3–30 mg/kg) or oral route (3–120 mg/kg) yielded ED_{50} (±95% confidence interval) values of 3.5 (2.1–4.8) and 89 (62–117) mg/kg, with peak inhibitory effect recorded after 5 min from i.v. administration and after 40 to 75 min from oral administration (time courses represented in Fig. 6). In the same model, ritodrine inhibited oxytocin-induced uterine contractions with an ED_{50} value of 45.2 (23.6–66.8) mg/kg

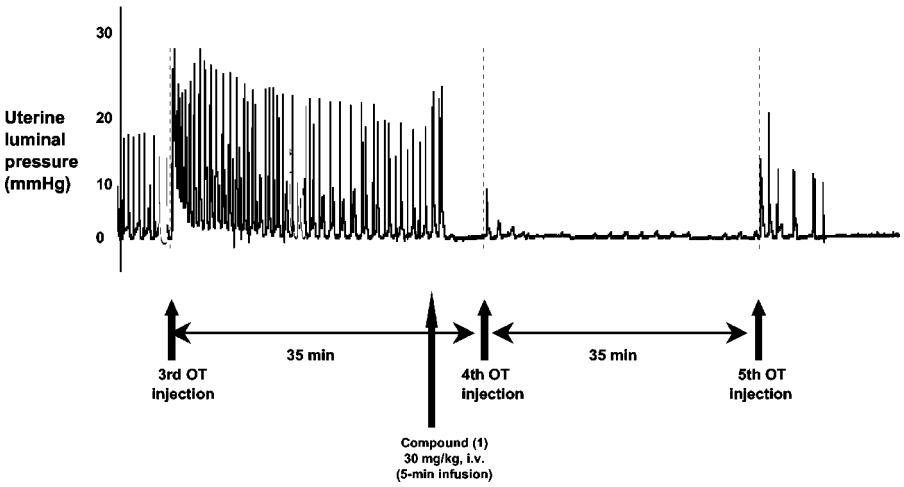

Fig. 5. Representative tracings of the inhibiting effects of compound **1** (30 mg/kg i.v. infusion) in oxytocin-induced uterine contractions in anesthetized nonpregnant rats.

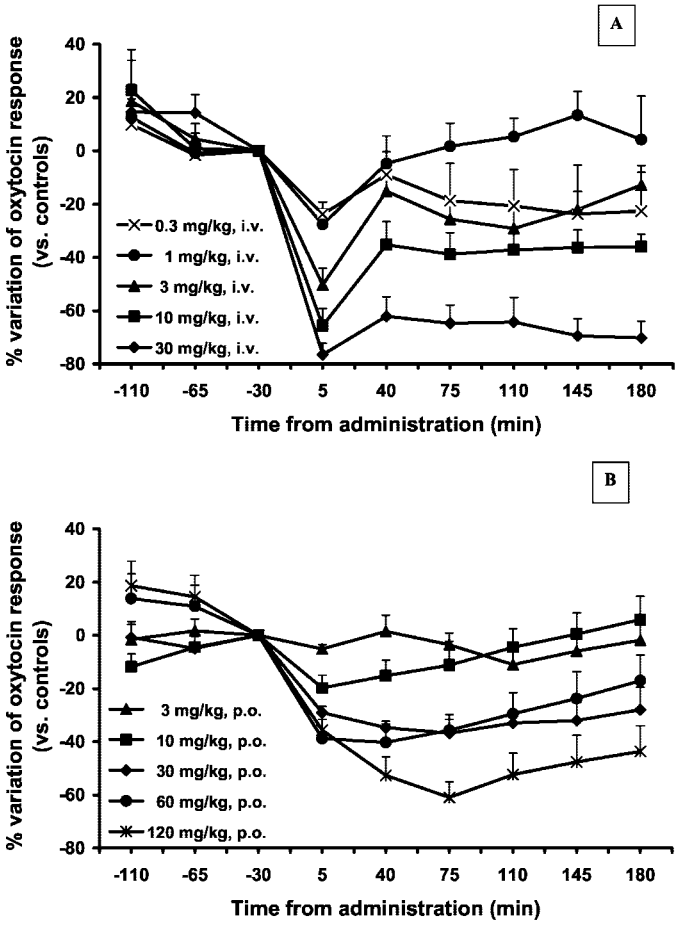

Fig. 6. Time course effects of compound **1** on oxytocin-induced uterine contractions in anesthetized rats by i.v. (A) or oral (B) routes. The data are presented as mean ± S.E. of at least $n = 6$ to 8 for treatment group. For A, significance is achieved with 1 ($p < 0.01$ at 5 min), 3 to 10 ($p < 0.001$ at 5 min), and 30 mg/kg i.v. ($p < 0.001$ at all time points). For B, significance is achieved with 30 ($p < 0.01$ at 5, 40, 75, and 110 min), 60 ($p < 0.001$ at 5 and 40 min, $p < 0.01$ at 75 min, and $p < 0.05$ at 110 min), and 120 mg/kg p.o. ($p < 0.001$ at all time points).

for the i.v. route and 51.9 (35.1–68.8) mg/kg for the oral route. The peak inhibitory effects for ritodrine were after 5 min after .v. administration and 75 min for oral route.

Pharmacokinetic Properties of Compound 1 in Female Rat. The absolute oral bioavailability of compound **1** in female conscious and nonpregnant rat was F = 49% at the dose of 50 mg/kg. The absorption was rapid with plasma concentrations of 763 ng/ml after 30 min and of 847 ng/ml at peak after 4 h. The half-life by i.v. route was $t_{1/2} = 2.8$ h.

Pharmacodynamics/Pharmacokinetics Correlations for Inhibition of Oxytocin-Induced Uterine Contractions in Anesthetized Nonpregnant Rats. Plasma concentrations after i.v. doses showed good dose-response linearity in the range from 0.3 to 10 mg/kg ($r = 0.995$). The correlation of the i.v. plasma levels with the observed inhibition of OT-induced uterine contraction (at peak inhibitory effect, namely, 0.033 h) indicated that an approximate 50% inhibition of OT contractions was achieved by this route when plasma concentration was about 1300 ng/ml (Fig. 7), i.e., at a dose of 3 mg/kg. Plasma levels after oral administration showed a poor dose-response linearity (Fig. 7) and the absolute bioavailability did not exceed 13%. From the pharmacodynamic point of view, the maximal inhibition by this route was less than 40% and was achieved when the concomitant plasma levels (at peak inhibitory effect, i.e., 1 h) accounted for about 400 ng/ml.

Effect on $PGF_{2\alpha}$-Induced Uterine Contractions in Anesthetized Nonpregnant Rats. Each administration of

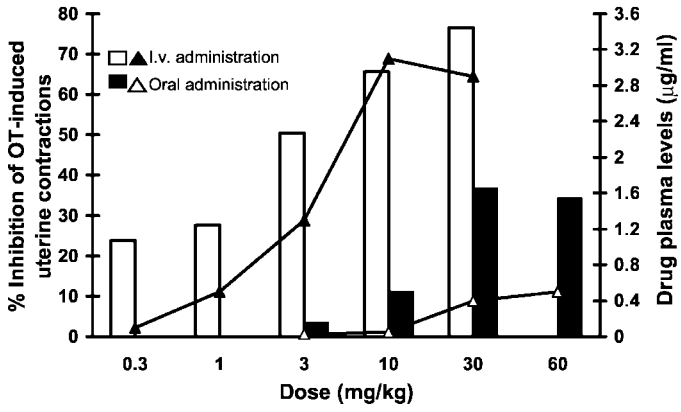

Fig. 7. Correlation between the inhibition of oxytocin-induced uterine contractions (open and solid columns, for the i.v. and oral administration, respectively) and plasma levels of compound **1** (solid and open triangles, for the i.v. and oral administration, respectively) in nonpregnant rats. Pharmacological effects and plasma levels were measured 5 min after the i.v. or 60 min after the oral administrations. Plasma levels are represented as the average obtained from pooling at least six plasma samples per dose.

$PGF_{2\alpha}$ caused a train of uterine contractions, mainly recorded as an increase in the intraluminal pressure and in frequency, which gradually declined in about 15 min after injection (data not shown). Repeated injections of $PGF_{2\alpha}$ resulted in reproducible uterine contractile responses and no major changes over time were observed when administering the vehicle alone (data not shown). Administration of increasing doses (3–30 mg/kg) of compound **1** by i.v. route produced a dose-dependent inhibition of $PGF_{2\alpha}$-induced uterine contractions (Fig. 8). Maximal inhibition rate (around 40%) was obtained at the dose of 30 mg/kg i.v. ($p < 0.001$ at 5-min time point). In the same model, ritodrine at 10 mg/kg i.v. inhibited $PGF_{2\alpha}$-induced uterine contractions with a maximal inhibition rate of ~50% ($p < 0.001$ and $p < 0.05$ at 5- and 40-min time points; Fig. 8).

Effect on Spontaneous Uterine Contractions in Anesthetized Late-Term Pregnant Rats. Spontaneous uterine contractions in late-term pregnant anesthetized rats were reasonably stable throughout the experimental period. Compound **1** at 1 and 3 ($p < 0.01$) mg/kg/min given during a 10-min infusion period markedly decreased contractile activity (Fig. 9A). The inhibitory effect seemed reversible, because it slowly declined in the 10 to 20 min of observation after the end of each infusion period (representative tracings in Fig. 10). In these administration conditions (10-min i.v. infusion) ritodrine seems to be already effective at a lower dose (0.3 mg/kg/min), but when administered as i.v. bolus at the same dosages, compound **1** was able to inhibit spontaneous uterine contractions more efficiently than ritodrine with significant inhibition of uterine activity with 1 ($p < 0.05$), 3 ($p < 0.001$), and 10 mg/kg i.v. ($p < 0.001$; Fig. 9B). Finally, compound **1** was also able to inhibit spontaneous uterine contractions in late-term pregnant anesthetized rats when given p.o. (Fig. 11). The maximal inhibition rate reached was ~20% for the higher dosage (100 mg/kg; $p < 0.05$ and $p < 0.01$ at 30/60 and 180 min, respectively) at around 10 min postadministration, whereas at the same dose ritodrine was ineffective (Fig. 11).

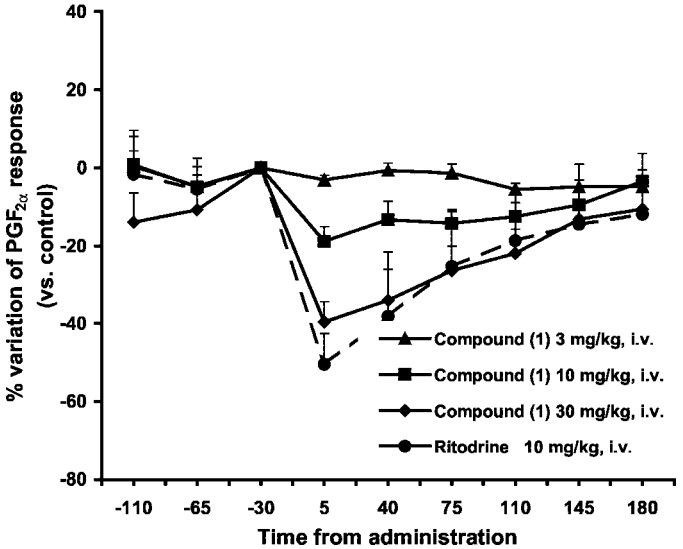

Fig. 8. Time course effects of compound **1** (3–30 mg/kg i.v.) or ritodrine 100 mg/kg i.v. on $PGF_{2\alpha}$-induced uterine contractions in anesthetized rats. The data are presented as mean ± S.E. of at least $n = 6$ to 8 for treatment group. *, $p < 0.05$ and ***, $p < 0.001$ versus vehicle-treated group (one-way ANOVA followed by Tukey's test).

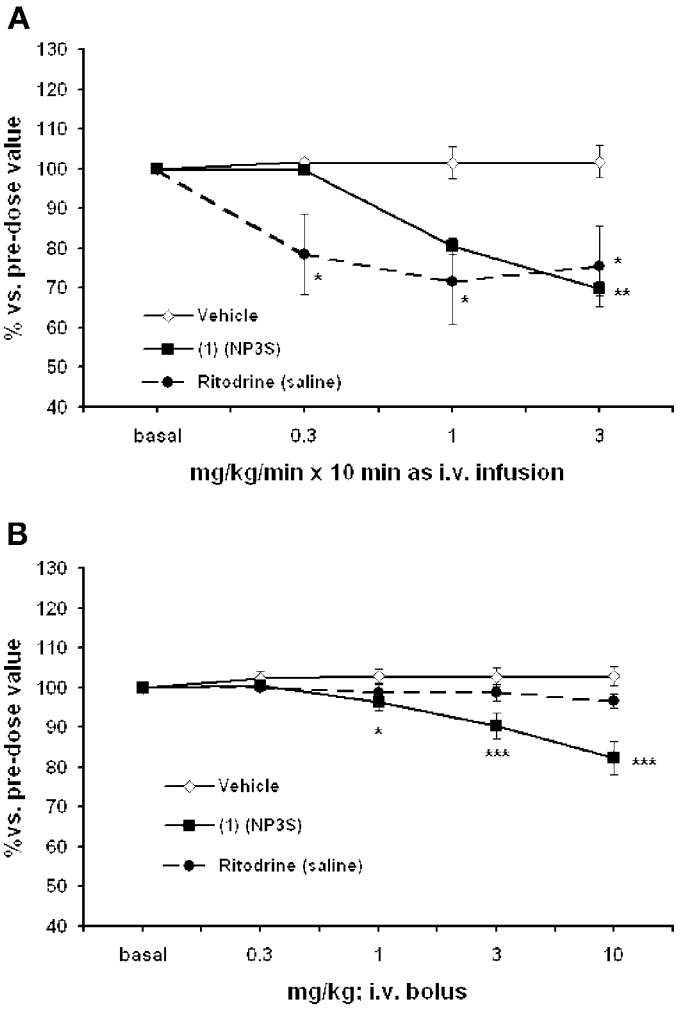

Fig. 9. Effects of increasing doses of ritodrine or compound **1** administered during 10 min i.v. infusion (A) or bolus i.v. (B) on spontaneous uterine contractions in anesthetized late-term pregnant rats. The data are presented as mean ± S.E. of at least $n = 6$ to 8 for treatment group. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus vehicle-treated group (one-way ANOVA followed by Tukey's test).

Discussion

The incidence of preterm birth has remained unchanged over the last decades. This is in part due to the complex etiology of preterm labor, and the limited ability of tocolytic agents to prolong pregnancy, as a consequence of their limited efficacy and poor safety profiles. Indeed, the clinical use of effective tocolytic agents such as β_2-adrenergic mimetics is limited by serious maternal and fetal side effects at cardiovascular levels, due to lack of selectivity against β_1-receptor.

The recent introduction of the OT-R antagonist atosiban represents a new generation of uterine-specific tocolytic agents, which are associated with more favorable safety profiles that would allow prolonged period of treatment at earlier or later gestational ages. The principal drawback to the use of peptide OT-R antagonists, like atosiban, is the lack of oral bioavailability due to intestinal degradation, that allow their use only by constant i.v. infusion, therefore limiting the treatment to only acute, threatened preterm labor for no more than 48 h.

In this article, we report on the discovery of compound **1**, a new, potent, and selective nonpeptide low molecular weight

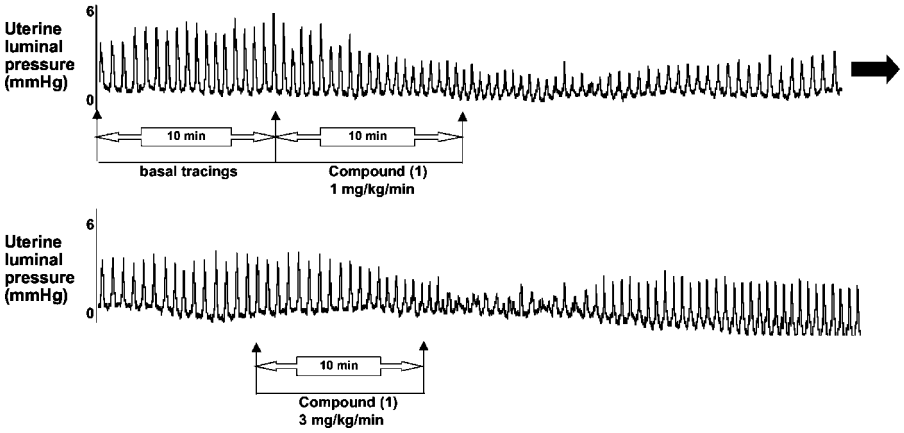

Fig. 10. Representative tracings of the effect of two different doses of compound **1** on spontaneous uterine contraction in pregnant rats near term (days 19–21). The lower tracings are a time continuation of the upper tracings.

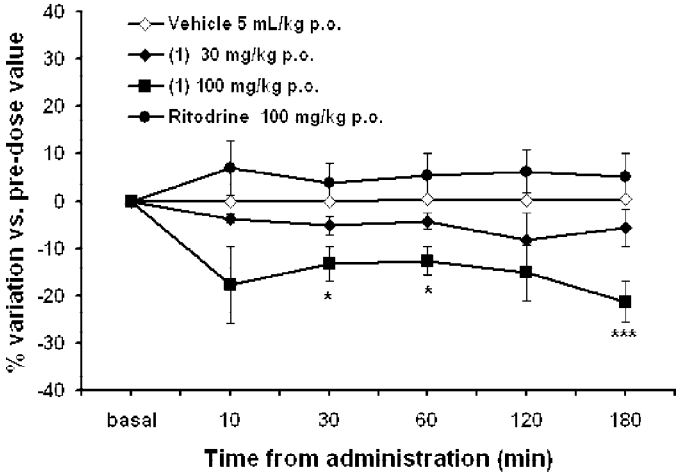

Fig. 11. Time-course effects of ritodrine (100 mg/kg) or increasing doses of compound **1** administered orally on spontaneous uterine contractions in anesthetized late-term pregnant rats. The data are presented as mean ± S.E. of at least $n = 6$ to 8 for treatment group. *, $p < 0.05$ and **, $p < 0.01$ versus vehicle-treated group (one-way ANOVA followed by Tukey's test.

OT-R antagonist. The in vitro binding profiling revealed high affinity of compound **1** for human and rat-OT-R within the range of atosiban. Moreover, compound **1** displayed selectivity against vasopressin receptor of >6-fold for V1a and >350-fold for V1b and V2 subtypes, whereas in humans, atosiban has higher affinity for V1a than for OT-R. Although AVP and vasopressin V1a receptor are present in the uterus it has been shown that they are not increased during labor (Thornton et al., 2002) and their antagonism is unlikely to contribute to reduction of uterine contraction at labor, or preterm labor (Thornton et al., 2001). On the contrary, long-term vascular V1a antagonism can induce unwanted maternal and fetal hypotensive side effects during tocolytic therapy (Chang et al., 1996).

Functional characterization on human OT-R performed in HEK293-EBNA cells showed that compound **1** dose dependently inhibited OT-induced intracellular calcium mobilization with an IC_{50} = 8 nM, 7 times more effectively than atosiban. When tested in nonstimulated cells, compound **1** presented no agonistic properties on calcium mobilization. This potent inhibitory effect on OT-induced cellular calcium mobilization was reflected in the antagonistic activity (pA_2 = 7.82) shown by compound **1** on OT-induced contraction of isolated rat uterine strips.

To investigate the pharmacological profile and selectivity of compound **1** in vivo, we used three different experimental models and compared the potency of our compound with the β_2-mimetic ritodrine (the only Food and Drug Administration-approved drug for the treatment of preterm labor).

Compound **1** dose dependently inhibited OT-induced uterine contractions in anesthetized nonpregnant rats with an ED_{50} value of 3.5 mg/kg for the i.v. route and 89 mg/kg for the oral route. The effect was marked and persistent. In the same experimental setup, ritodrine was ~10 times less potent when administered i.v. (ED_{50} = 45.2 mg/kg), and displayed similar, or slightly higher, potency when given orally (ED_{50} = 51.9 mg/kg). In this model, there was a good correlation between compound **1** plasma levels and the pharmacological effect, indicating that ~50% inhibition of OT-induced uterine contractions is achieved at plasma levels of 1.3 μg/ml. The bioavailability after oral dosage was estimated to be 13%. However, these estimations could have been affected by the experimental conditions (e.g., anesthesia, treatment with diethylstilbestrol) that may affect the pharmacokinetic profile. Indeed, oral bioavailability was much higher (49%) in conscious female rats, thus suggesting that compound **1** could become an oral tocolytic agent in the clinic. However, because bioavailability could differ in some cases between animal species and humans, only future clinical studies in humans will ascertain the human bioavailability of compound **1** and its potential as a tocolytic drug.

The selectivity of compound **1** in inhibiting specifically OT-induced contraction was tested in vivo in anesthetized nonpregnant rats, in which uterine contractions were induced by i.v. injections of $PGF_{2\alpha}$. In this model, compound **1**, administered i.v. at 3 mg/kg (i.e., ~ED_{50} in the OT-induced contraction model), was unable to inhibit uterine contraction. Increasing the dose up to 30 mg/kg induced about 40% inhibition of the $PGF_{2\alpha}$-induced uterine contractions; the same effect was observed with ritodrine at 10 mg/kg.

Finally, because it seems highly likely that OT plays an important role at a uterine level, in the initiation and progression of uterine contractions that result in labor, both in rodents and humans (Gimpl and Fahrenholz, 2001), we challenged compound **1** on spontaneous uterine contraction in anesthetized pregnant rats near to term (days 19–21 of pregnancy). We adopted three different administration para-

digms, i.v. by 10-min infusion, bolus i.v., and oral. After i.v. dosage, compound **1** was able to dose dependently inhibit spontaneous uterine contraction by ~25%, with comparable inhibitory capacity when administered as a bolus or by slow infusion. On the contrary, ritodrine was more effective when administered by 10-min infusion and lost most of its inhibitory ability if administered as bolus. Also, when administered orally the inhibitory ability of compound **1** was maintained, whereas in this condition and at equal dosage ritodrine was totally ineffective.

In summary, we have reported the discovery of a small molecule nonpeptide OT-R antagonist, active on ex vivo and in vivo models of uterine contractions. Compound **1** has high affinity and specificity for human OT receptor and is very selective against V1a, V1b, or V2 receptor subtypes. The 5-fold higher affinity for human versus the rat OT receptor in the in vitro study promises for higher potency in humans. Moreover, Compound **1** is suitable for oral use, an advantage over the peptide OT-R receptor antagonist atosiban, at present marketed in Europe as tocolytic for clinical use in the management of threatened preterm birth. In fact, although i.v. efficacy is attractive for the rapid onset of action in the control of preterm labor, oral bioavailability would be needed for long-term maintenance use and possibly prophylaxis in women at risk of preterm labor.

In the past, other groups have identified nonpeptide OT-R antagonists with very interesting in vitro and in vivo profiles (Clineschmidt et al., 1991; Pettibone et al. 1993; Williams et al., 1998), but no reports are available on the current stage of the development of these compounds. The need to identify new selective and nonpeptide oxytocin receptor antagonist therefore remains. Compound **1** seems to have sufficiently promising pharmacological features to become a potential candidate for the management of preterm uterine hyperactivity.

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